

## Microbiological Assay and HPLC Method for the Determination of Fluconazole in Pharmaceutical Injectable Formulations

Felipe K. HURTADO, Marinês J. SOUZA, Janine de MELO & Clarice M.B. ROLIM \*

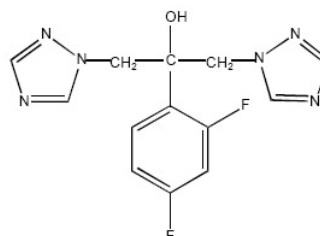
Department of Industrial Pharmacy, Health Science Center,  
Federal University of Santa Maria,  
97.105-900 - Santa Maria, RS, Brazil

**SUMMARY.** Fluconazole is a synthetic triazole antifungal agent used in the treatment of candidiasis and other fungal infections. A high performance liquid chromatographic method and a microbiological assay have been developed for the determination of fluconazole in injectable solutions. A Phenomenex Synergi Fusion RP-80 C<sub>18</sub> (150 x 4.60 mm, 4 μm) column was used for fluconazole separation, using isocratic elution with water: methanol (55:45, v/v) and UV detection at 260 nm. Microbiological assay (bioassay) was performed using the agar diffusion method, using *Saccharomyces cerevisiae* ATCC 1600 as the test microorganism and antibiotic medium 19 for agar layer. The two methods were validated and applied for quantitative determination of fluconazole and have demonstrated good linearity, precision and accuracy. The assays were linear in the concentration range of 25-200 μg ml<sup>-1</sup>, for HPLC, and 25-400 μg ml<sup>-1</sup> for microbiological method. The precision of the methods was determined by repeatability (RSD 0.18%, for HPLC, and 3.0% for bioassay) and intermediate precision (RSD 0.32%, for HPLC, and 4.73% for bioassay). The accuracy was determined and the mean recovery was found to be 99.25%, for HPLC, and 98.89% for bioassay. The two proposed methods have been successfully validated and may be considered for routine analysis of fluconazole in pharmaceutical injectable preparations.

**RESUMEN.** “Ensayo Microbiológico y Método por HPLC para la Determinación de Fluconazol en Formulaciones Farmacéuticas Inyectables”. Fluconazol es un agente antifúngico triazólico sintético empleado en el tratamiento de la candidiasis y de otras infecciones fúngicas. Un método por cromatografía líquida de gran eficacia y un ensayo microbiológico fueron desarrollados para la determinación de fluconazol en soluciones inyectables. Una columna Phenomenex Synergi Fusion RP-80 C<sub>18</sub> (150 x 4.60 mm, 4 μm) fue usada para la separación, utilizando elución isocrática con agua: metanol (55:45, v/v) y detección por ultravioleta a 260 nm. El ensayo microbiológico (bioensayo) fue realizado utilizando el método por difusión en agar, empleando *Saccharomyces cerevisiae* ATCC 1600 como microorganismo test y medio antibiótico 19 como medio de cultivo. Los dos métodos resultaron válidos y fueron aplicados para la determinación cuantitativa de fluconazol, demostrando buena linealidad, precisión y exactitud. Los ensayos fueron lineales en la franja de concentración de 25-200 μg ml<sup>-1</sup>, para el método por HPLC y 25-400 μg ml<sup>-1</sup>, para el método microbiológico. La precisión de los métodos fue determinada como repetibilidad (DPR 0,18%, por HPLC y 3,0% por el bioensayo) y precisión intermedia (DPR 0,32%, por HPLC y 4,73% por el bioensayo). La exactitud fue determinada y la recuperación media encontrada fue 99,25%, por HPLC y 98,89% por el bioensayo. Los dos métodos propuestos resultaron válidos y pueden ser empleados para el análisis de rutina de fluconazol en preparaciones farmacéuticas inyectables.

### INTRODUCTION

The increase in the incidence of fungal infections may be attributed primarily to an increase in the number of individuals at risk for these infections<sup>1</sup>. Fluconazole (Fig. 1) is a triazole antifungal agent widely used in clinical practice, and has a broad range of applications in the treatment of both superficial and systemic mycoses<sup>2</sup>. The favorable pharmacokinetics of fluconazole (high oral bioavailability, minimal metabolism, low protein binding in plasma, and



**Figure 1.**  
Chemical structure of fluconazole.

predominant renal excretion as unchanged drug) facilitate the management of its dosing<sup>3</sup>. Its applications include the treatment of *Candi-*

**KEY WORDS:** Fluconazole; Injectable solutions; HPLC; Microbiological assay  
**PALABRAS CLAVE:** Fluconazol; Soluciones inyectables; HPLC; Ensayo microbiológico

\* Author to whom correspondence should be addressed. E-mail: crolim@smail.ufsm.br

da infections and the meningitis caused by *Cryptococcus neoformans*, typical diseases of immunocompromised individuals.

Some analytical methods have been published for fluconazole determination in pharmaceutical formulations using liquid chromatography<sup>4</sup> and spectrophotometry<sup>5-8</sup>. Several procedures have been described for the determination of fluconazole in plasma: chromatographic<sup>9-13</sup> and microbiological<sup>2,14</sup> methods. However, the microbiological assay for determination of fluconazole in pharmaceuticals has not yet been reported.

In this paper, a simple, rapid and economical HPLC method and a new microbiological assay have been developed and validated for quantitative determination of fluconazole in pharmaceutical injectable preparations.

## MATERIALS AND METHODS

Fluconazole standard substance (assigned purity 99.28%) was obtained from DEG (São Paulo, Brazil). Samples of fluconazole injection 200 mg bags containing 2 mg ml<sup>-1</sup> in sodium chloride and water were obtained from commercial sources from the respective manufacturers, and used within their shelf life period. All solvents were HPLC grade, and all reagents were of analytical grade. Methanol was obtained from Tedia (Fairfield, USA). Water was purified with WaterPro™ PS, Labconco system (MO, USA). All solvents were filtered through a 0.45 µm nylon membrane filter (Whatman, USA) and degassed before use. The solutions were filtered through 0.22 µm Millex-HV filter units (Millipore, USA) before injection.

### HPLC method

Chromatography was performed on a Shimadzu (Kyoto, Japan) HPLC system, consisting of two model LC-10ADVP pumps, and a model SIL-10AF auto-sampler, equipped with UV-vis detector model SPD-10AVP. Data integration was performed using Shimadzu Class-VP software. Fluconazole was separated on a reversed phase Phenomenex (CA, USA) Synergi Fusion-RP 80 C<sub>18</sub> (150 x 4.60 mm i.d., 4 µm particle size) analytical column. All analyses were done at room temperature (24 ± 2 °C) under isocratic conditions. The mobile phase consisted of a mixture of water: methanol (55:45, v/v). The flow rate was 1.0 ml min<sup>-1</sup> and the volume of injection was 20 µl. The UV detection was set at 260 nm.

## Microbiological assay

### Organism and inoculum

The cultures of *Saccharomyces cerevisiae* ATCC 1600 (INCQS, Rio de Janeiro, Brazil) were cultivated on antibiotic medium 19 (Difco, USA), maintained in the refrigerator and transferred to another antibiotic medium 19 (24 h before the assay) which was kept in incubator at 35 ± 1 °C. The microorganisms were suspended in sterile NaCl 0.9%. Diluted suspension cultures of 25 ± 2% turbidity were obtained at 580 nm, using a suitable spectrophotometer (Bausch & Lomb, USA) and a 13 mm diameter test tube as an absorption cell against NaCl 0.9% as blank. Portions of 1 ml of the inoculated NaCl 0.9% were added to 100 ml of antibiotic medium 19 at 45 ± 2 °C and used as inoculated layer.

### Assay

The antibiotic medium 19 (15 ml) was placed into 100 mm x 20 mm Petri dishes for the agar layer. After solidification of this layer, six stainless steel cylinders were placed on the surface of inoculated medium. Three alternated cylinders were filled with 100 µl of the standard solutions and the other three with the sample solutions. After incubation (35 °C for 24 h) the zone diameters (in mm) of the growth inhibition were measured using a digital caliper (Starret, USA). Eight plates were used for each assay.

### Preparation of the fluconazole standard solution

For HPLC method, 25 mg of fluconazole standard substance was transferred to a 25 ml volumetric flask and 10 ml of methanol was added. The flask was sonicated during 5 min, followed by sufficient quantity of methanol to obtain 1000 µg ml<sup>-1</sup>. The resulting solution was diluted in mobile phase to obtain a final concentration of 100 µg ml<sup>-1</sup>. All solutions were prepared fresh daily.

For microbiological assay, 10 mg of fluconazole standard substance was transferred to a 10 ml volumetric flask and 2 ml of methanol was added. The flask was sonicated during 5 min, followed by sufficient quantity of distilled water to obtain 1000 µg ml<sup>-1</sup>. The resulting solution was diluted in distilled water to obtain 100 µg ml<sup>-1</sup>.

### Preparation of the sample

For HPLC method, a volume of injectable solution equivalent to 20 mg of fluconazole was transferred to a 20 ml volumetric flask, followed

by sufficient quantity of mobile phase. The resulting solution was diluted in mobile phase to obtain 100  $\mu\text{g ml}^{-1}$ .

For microbiological assay, a volume of injectable solution equivalent to 20 mg of fluconazole was transferred to a 20 ml volumetric flask, followed by sufficient quantity of distilled water. The resulting solution was diluted in distilled water to achieve final concentrations of 25, 100 and 400  $\mu\text{g ml}^{-1}$ .

### Method validation

The methods were validated according to the International Conference on Harmonisation guidelines for validation of analytical procedures<sup>15</sup>. Analysis of variance (ANOVA) was used to verify the validity of the methods.

#### Linearity

For HPLC method, the calibration curve was obtained with six concentrations of the standard solution (25-200  $\mu\text{g ml}^{-1}$ ). For microbiological assay, the calibration curve was obtained with three concentrations of the standard solution (25-400  $\mu\text{g ml}^{-1}$ ). The solutions were prepared and analyzed in three different days. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

#### Precision

The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Intra-day precision was evaluated by assaying samples at the same concentration and on the same day. Six sample solutions (100  $\mu\text{g ml}^{-1}$ ) were prepared and assayed. The intermediate precision was studied by comparing the assays on different days (three days).

#### Accuracy

The accuracy was evaluated by the assay of three concentrations of the sample solution (80, 100 and 120  $\mu\text{g ml}^{-1}$ , for HPLC method; 75, 150, and 300  $\mu\text{g ml}^{-1}$ , for microbiological assay) in

triplicate. The solutions were prepared and analyzed in three different days.

#### Robustness

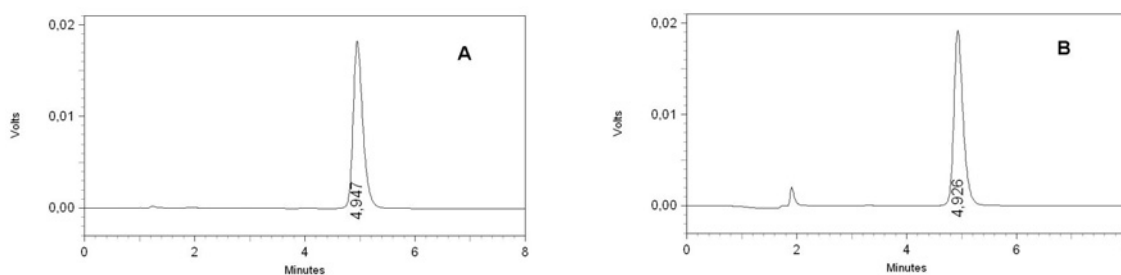
The robustness of the HPLC method was determined by the analysis of the samples under a variety of conditions by making small changes in the percentage of methanol (5%) in the mobile phase, in the flow rate (0.8-1.2  $\text{ml min}^{-1}$ ) and by changing the chromatographic column.

## RESULTS AND DISCUSSION

### HPLC method

A simple and rapid reversed-phase HPLC method was proposed for the quantitative determination of fluconazole in pharmaceutical injectable dosage form. Based on physical-chemical characteristics of fluconazole, the proposed method employed a  $\text{C}_{18}$  chromatographic column. The retention time observed (4.9 min) allowed a rapid determination of the drug. The mobile phase is easy to prepare and uses low-cost solvents, such as water and methanol. Fig. 2 shows a representative chromatogram obtained from the analysis of a standard and a sample solution of fluconazole using the proposed method. As shown in this figure, fluconazole formed a symmetrical peak, well separated from the solvent front.

The calibration curves for fluconazole were constructed by plotting concentration *versus* peak area and showed good linearity in the 25-200  $\mu\text{g ml}^{-1}$  range. The representative linear equation was  $y = 2019.5x + 1550$ , with a highly significant correlation coefficient ( $r = 0.9999$ ) for the method. The inter and intra-day precision were calculated and showed a RSD of 0.32 and 0.18%, respectively. The accuracy was 99.25%, and was calculated by the agreement between the theoretical value and the value found. The results and the mean values demonstrating good precision and accuracy are shown in Table 1. The experimental values obtained for the determination of fluconazole are presented in Table



**Figure 2.** Chromatograms of fluconazole 100  $\mu\text{g ml}^{-1}$ , (A) standard and (B) sample. Phenomenex™  $\text{C}_{18}$  column (150 x 4.60 mm i.d., 4  $\mu\text{m}$ ). Water: methanol (55:45, v/v) as mobile phase. Flow rate 1.0  $\text{ml min}^{-1}$ . 260 nm.

Theoretical concentration ( $\mu\text{g ml}^{-1}$ )		Intra-day		Inter-day	
HPLC	Accuracy <sup>a</sup> (%)	Precision <sup>a</sup> (RSD%)	Accuracy <sup>a</sup> (%)	Precision <sup>a</sup> (RSD%)	
80	100.12	0.13	100.30	0.38	
100	98.45	0.17	98.14	0.29	
120	99.20	0.24	98.70	0.29	
Bioassay	Accuracy <sup>b</sup> (%)	Precision <sup>b</sup> (RSD%)	Accuracy <sup>b</sup> (%)	Precision <sup>b</sup> (RSD%)	
75	98.82	4.31	100.34	6.20	
150	99.55	2.81	100.03	5.12	
300	98.32	1.89	102.1	2.87	

**Table 1.** Intra-day and inter-day accuracy and precision data of HPLC and microbiological methods for fluconazole. <sup>a</sup> Mean of three determinations for each concentration. <sup>b</sup> Mean of eight determinations for each concentration.

Method	Sample ( $\mu\text{g}$ )	Experimental amount (%)	[ $\text{mg (100 ml)}^{-1}$ ]	RSD (%)
HPLC	100	101.43 <sup>a</sup>	202.87	0.35
		101.61 <sup>a</sup>	203.23	
		100.92 <sup>a</sup>	201.85	
Bioassay	100	103.75 <sup>b</sup>	207.50	1.14
		101.86 <sup>b</sup>	203.72	
		101.60 <sup>b</sup>	203.20	

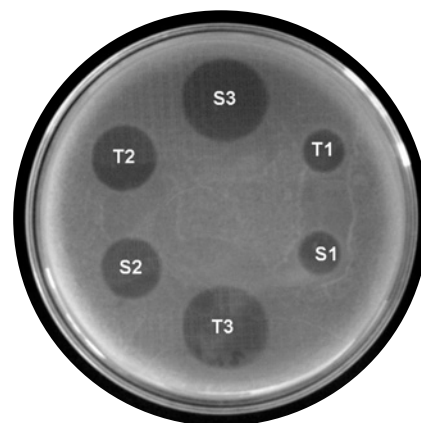
**Table 2.** Results of the determination of fluconazole in injectable solution by HPLC and microbiological methods. <sup>a</sup> Mean of three determinations. <sup>b</sup> Mean of eight determinations.

2. Deliberate variation on the chromatographic conditions had no significant effect on assay results or on chromatographic performance, confirming the robustness of the method.

The proposed method has an advantage when compared with the method described by literature <sup>4</sup>, which used trishydroxymethyl aminomethane in phosphate buffer in the mobile phase. Buffers decrease the shelf life of chromatographic columns. Besides, the proposed HPLC method was validated and successfully applied for the determination of fluconazole in injectable preparations.

#### Microbiological assay

In this work, an experimental 3 x 3 design agar diffusion bioassay, using three dose levels for each standard and sample, was used following the procedure described in the Brazilian Pharmacopoeia <sup>6</sup>. The calculation procedure usually assumes a direct relationship between the observed zone diameter and the logarithm of the applied dose. The corresponding mean zone diameters for fluconazole standard solution were: 12.25 mm (RSD = 8.73%) for low dose, 16.81 mm (RSD = 6.08%) for medium dose and 21.91 mm (RSD = 3.30%) for high dose (Fig. 3). The calibration curves for fluconazole were constructed by plotting log of con-



**Figure 3.** Plate obtained from the microbiological assay (agar diffusion method). Fluconazole standard at concentrations 25 (S1), 100 (S2) and 400  $\mu\text{g ml}^{-1}$  (S3) and fluconazole injection at concentrations 25 (T1), 100 (T2) and 400  $\mu\text{g ml}^{-1}$  (T3), using a strain of *Saccharomyces cerevisiae* ATCC 1600 as the test microorganism.

centration ( $\mu\text{g ml}^{-1}$ ) versus zone diameter (mm) and showed good linearity in the 25-400  $\mu\text{g ml}^{-1}$  range. The representative linear equation was  $y = 9.2163x + 0.9241$ , where x is the log dose and y the diameter zone. The correlation coefficient was  $r = 0.9998$ .

The experimental values obtained for the determination of fluconazole in injectable dosage form are presented in Table 2. The precision

Amount labelled (100%)	HPLC (%)	Bioassay (%)
Amount found*	100.88	100.81
RSD	0.54	1.94

**Table 3.** Comparison of the two methods for the determination of fluconazole in injectable formulation, ANOVA test. \* Average of six experiments. ANOVA, ( $p < 0.01$ ).  $F_{\text{calc}} = 0.00711$ ;  $F_{\text{tab}} = 5.18$ .

and accuracy of the method were studied. The inter and intra-day precision obtained by the assay showed a RSD of 4.73 and 3.0%, respectively. The accuracy was found to be 98.89%. The results are expressed in Table 1.

The quantification of antimicrobial drugs by chemical methods such as HPLC and spectrophotometry, although precise, cannot provide a true indication of biological activity<sup>16</sup>. The potency of antibiotics may be demonstrated under suitable conditions by their inhibitory effect on microorganisms. A reduction in antimicrobial activity may also reveal subtle changes not demonstrable by chemical methods<sup>17</sup>. This assay has the advantages of using simple apparatus and aqueous solvents, reducing the generation of chemical residues.

The proposed analytical methods were compared using statistical analysis. ANOVA was applied and did not reveal a significant difference between the experimental values obtained by the two methods. The calculated F-value ( $F_{\text{calc}} = 0.00711$ ) was found to be less than the tabled F-value ( $F_{\text{tab}} = 5.18$ ) at a 1% significance level (Table 3).

## CONCLUSION

The results indicated that the proposed methods demonstrated good linearity, precision and accuracy. Analytical results of the samples were in accordance with those of the standard solution at the same concentrations. The methods use simple reagents, with minimum sample preparation procedures. Both methods enable a quantitative determination of fluconazole in pharmaceutical injectable preparations and can be used in routine analysis.

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